

Review Article

Cholinergic regulation of bone

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Abstract

Bone remodeling is regulated by the two branches of the autonomic nervous system: the adrenergic and the cholinergic branches. Adrenergic activity favors bone loss, whereas cholinergic activity has been recently shown to favor bone mass accrual. *In vitro* studies have reported that cholinergic activity induces proliferation and differentiation of bone cells. *In vivo* studies have shown that the inhibition of cholinergic activity favors bone loss, whereas its stimulation favors bone mass accrual. Clinical studies have shown that bone density is associated with the function of many cholinergic-regulated tissues such as the hypothalamus, salivary glands, lacrimal glands and langerhans cells, suggesting a common mechanism of control. Altogether, these observations and linked findings are of great significance since they improve our understanding of bone physiology. These discoveries have been successfully used recently to investigate new promising therapies for bone diseases based on cholinergic stimulation. Here, we review the current understanding of the cholinergic activity and its association with bone health.

Keywords: Cholinergic, Parasympathetic Nervous System, Bone, Acetylcholine, Osteoporosis

Introduction

Bone remodeling involves bone resorption by osteoclasts and subsequent formation of new bone by osteoblasts¹⁻³. This process regulates the biomechanical features of bone and maintains the homeostasis of essential mineral ions in the body¹. Alteration in the bone remodeling process results in diseases such as osteoporosis that can often cause life-threatening complications³. The mechanisms regulating bone remodeling are not fully understood, and unveiling them should eventually allow development of new therapeutic approaches for osteoporosis and other bone related diseases³.

The process of bone formation and resorption is well-regulated, at least, at two different levels: locally and centrally¹. Locally, bone remodeling is regulated through direct interaction between osteoblasts and osteoclasts, and by local interactions between cells of the immune system and bone cells¹. Centrally,

bone remodeling has been shown to be regulated at three axis: 1) the hypothalamic-pituitary-thyroid axis; 2) the co-regulation of bone, adipose tissue and energy metabolism mediated by the sympathetic nervous system (SNS) and 3) the IL 1- parasympathetic nervous system (PSNS)- bone axis⁴⁻¹¹. The last two axes are mediated by adrenergic and cholinergic activities, the functional arms of autonomic nervous system⁴⁻¹¹.

The adrenergic activity of the SNS has been shown to be a negative regulator of bone mass; adrenergic signaling inhibits osteoblast proliferation and promotes osteoclastogenesis^{4-6,12}. Moreover, SNS signaling induces the release of the osteoblast-derived soluble factor receptor activator of NF- κ B ligand (RANKL), which in turn augments osteoclastogenesis and bone resorption⁶. SNS signaling are well controlled by the action of two key proteins: osteocalcin and leptin. Osteocalcin, produced by mature osteoblasts and osteocytes, regulates the production of leptin, an adipocyte-derived hormone. Leptin, in-turn, activates its receptors in the hypothalamus and promotes SNS activity⁴⁻⁶.

Cholinergic activity has been shown to favor bone mass by increasing osteoblast proliferation via suppression of the SNS and promoting the apoptosis of osteoclasts^{10,11}. The present article reviews the current understanding of the cholinergic effect on bone remodeling based on a variety of *in vitro*, *in vivo* and clinical studies, and suggests new ways for preventing bone fractures and osteoporosis by cholinergic stimulation.

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Cholinergic system

Cholinergic system regulates the body's metabolic activity through the use of acetylcholine as a signal transmitter¹³. Acetylcholine is biosynthesized by choline acetyl transferase, and it is stored in small synaptic vesicles through the action of vesicular acetylcholine transporter enzyme to be released via exocytosis into the synapse space¹⁴. The secreted acetylcholine targets either nicotinic or muscarinic receptors^{15,16}. Nicotinic receptors are comprised of different subunits α , β , γ , δ and ϵ that assemble to form ionic channels¹⁷. Muscarinic receptors are guanine nucleotide protein coupled receptors, and are classified into five subtypes: m1, m2, m3, m4 and m5^{16,18}. Acetylcholine signal is terminated by its degradation through the enzyme acetylcholinesterase (AChE)¹⁹.

Many neuronal cells such as all pre- and post-ganglionic nerves of the PSNS, preganglionic and some postganglionic nerves of the SNS, motor neurons and neurons within the central nervous system express the components of the cholinergic system (transmitter, enzymes and receptors)^{10,11,20-22}. Moreover, many non-neuronal cells such as embryonic stem cells, epithelial cells and bone cells have been shown to express components of the cholinergic system as well^{13,23,24}.

Expression of cholinergic components in bone

Bone cells have been shown to express several components of the cholinergic system (transmitter, enzymes and receptors)^{10,11,15,16,25-30}. For instance, acetylcholine has been suggested to be produced by osteoblasts due to the presence of the vesicular acetylcholine transporter enzyme in these cells³⁰. Cholinergic receptors, both nicotinic and muscarinic, have been identified on the membranes of human primary bone cells, mesenchymal stem cells, osteoblasts and osteoclasts^{10,11,25,26,31-34}. The mRNA of several nicotinic receptors subtypes (α_1 , α_4 , β_1 and γ) are also present in osteocytes³⁵. AChE is expressed in bone cells such as bone marrow-derived monocytes, osteoclasts and osteoblasts^{11,29,36}.

Possible role of cholinergic components in bone

The wide expression of cholinergic components in bone tissue points toward the important role they could play in bone remodeling²³. Previous studies have shown that acetylcholine might regulate the migration of bone marrow-derived multiprogenitor mesenchymal stem cell³⁷ capable of differentiating into bone cells. Both families of cholinergic receptors, nicotinic and muscarinic, have been shown to affect bone turnover. Indeed, nicotinic stimulation in mice induces bone mass gain as a result of an increase in osteoclasts apoptosis¹¹, whereas muscarinic stimulation *in vitro* increases osteoblasts proliferation^{25,30}. Among the cholinergic receptors, the nicotinic subtype- α_2 receptor and muscarinic-3 receptor appear important in bone physiology^{10,11,38-40}. Indeed, mice with knockout nicotinic subtype- α_2 receptors are osteoporotic due to the up-regulation of osteoclasts¹¹, whereas mice with knockout muscarinic-3 receptors are osteoporotic due to a decrease of osteoblast numbers and an increase of osteoclast numbers¹⁰.

Beside its capability to break down acetylcholine, AChE is thought to play significant roles during remodeling of bone. For instance, AChE has been detected at the sites of new bone formation³⁶, suggesting a role for AChE as a bone matrix protein^{29,36}. Moreover, it has been reported that AChE can regulate bone cells proliferations, differentiations, cell-cell contact as well as mesenchymal stem cells migration^{27,29,36,41,42}.

Cholinergic innervation of bone

It has been shown that bone tissues are innervated by cholinergic fibers of both the PSNS and the SNS^{11,43}. Cholinergic fibers transmit neuronal signaling from the PSNS nucleus within the CNS to cholinergic receptors located in bone¹¹. Accordingly, it would be logical to expect that disruption in the function of these cholinergic fibers would affect bone. Indeed, it has been shown that mice subjected to subdiaphragmatic sectioning of the vagus nerve, a cranial nerve that carries cholinergic fibers of the PSNS, suffer from low bone mass in their lumbar vertebra⁴⁴. Cholinergic fibers of the SNS have been shown to innervate the periosteum, a connective tissue that covers the bone and contains progenitor cells that develop into osteoblasts, indicating a possible role of these neuronal fibers in regulating bone formation and remodeling⁴⁵. Indeed, denervation of the periosteum results in poor bone healing in animal models, indicating that periosteal nerves are required for bone formation and fracture healing⁴⁶⁻⁴⁹.

Central nervous system nuclei

Hypothalamus

Previous studies have shown that the hypothalamus, a brain structure that encloses cholinergic (muscarinic and nicotinic) and adrenergic components, can affect bone remodeling^{50,51}. It has been shown that Alzheimer's disease (AD) patients suffering from cholinergic degradation of the hypothalamus^{52,53}, are more prone to develop osteoporosis and suffer from a high incidence of fractures⁵⁴⁻⁵⁷. In fact, the association between bone mineral density and cholinergic degradation of the hypothalamus is so strong^{58,59} that decrease in bone mineral density has been suggested as a predictor of AD⁶⁰. These observations highlight the importance of cholinergic nucleus of the hypothalamus in regulating bone mass accrual.

One mechanism by which the hypothalamus might regulate bone remodeling is by controlling body weight⁴, a known positive regulator of bone mass^{61,62}. The hypothalamus encloses two structures that regulate body weight: the ventromedial and the lateral hypothalamic nuclei. The lateral hypothalamic nucleus is concerned in hunger, and any damage to this area can reduce body weight⁶³. Lateral hypothalamic nucleus is known to enclose cholinergic neurons and receptors, such as muscarinic receptors^{10,64}. Accordingly, suppression of cholinergic neurons activity in the lateral hypothalamic nucleus could explain why AD patients usually suffer from weight loss, hence low bone mass accrual⁶⁵. However, future research has to be done to test these hypotheses.

It is well known that the hypothalamus regulate bone mass also through a neurohormonal pathway mediated by the pitu-

Body tissue that expresses cholinergic receptors	Function of local cholinergic receptors	Effect of inhibition of local cholinergic receptors	Association between local inhibition of cholinergic receptors and bone mineral density (BMD)
Salivary glands ⁷⁰	Saliva secretion ⁷⁸	Dry mouth ⁷⁸	Patients with dry mouth are associated with low BMD ⁸¹
Lacrimal glands ⁷²	Lacrimal secretion ¹¹⁴	Dry eyes ⁷⁹	Patients with dry eyes are associated with low BMD ⁸²
Pancreas ⁷³	Insulin secretion ¹¹⁵	Diabetes type ^{180,116}	Patients with diabetes type 1 are associated with low BMD ^{83,84,117,118}
Respiratory system ¹¹⁹	Smooth muscle contraction and epithelial regulation ^{119,120}	Obstructive pulmonary disease ⁸⁸	Patients with lung diseases are associated with low BMD ⁸⁵
Gastrointestinal system ⁷⁴	Smooth muscle contraction ⁷⁴	Decrease muscle tone ¹²¹	Patients with gastrointestinal diseases are associated with low BMD ⁸⁹
Vestibular organ within ear ⁷⁶	Regulate balance and orientation ¹²²	Unbalance and disorientation (Vertigo)	Patients with vertigo are associated with low BMD ⁹²

Table 1. The relationship between cholinergic receptors in body tissues with bone turnover in various clinical conditions.

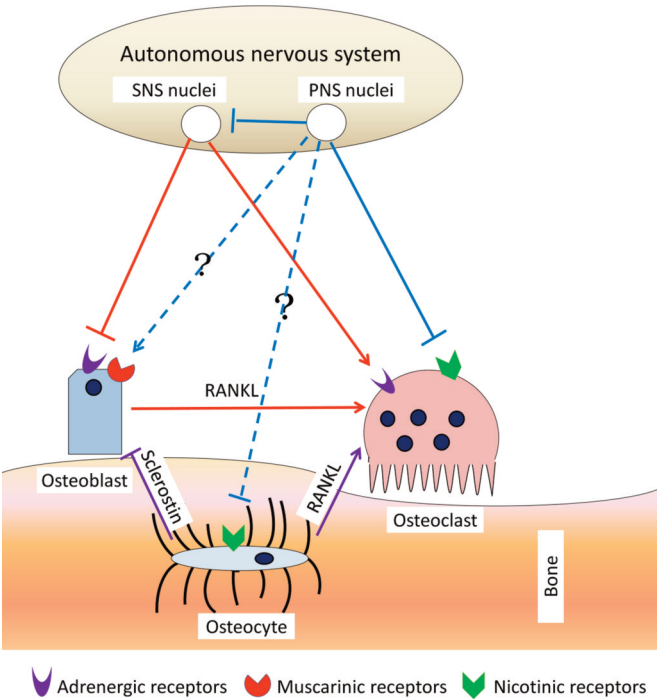


Figure 1. Model of the autonomic nervous system-mediated regulation of bone mass accrual. The SNS favors bone loss by inhibiting osteoblast proliferation, directly by activating the adrenergic receptors. Also, the SNS favors bone loss by promoting osteoclast proliferation, directly through activating the adrenergic receptors on osteoclasts and indirectly through stimulating RANKL secretion from osteoblasts. The PNS favors bone mass indirectly by suppressing the SNS signaling and directly by promoting apoptosis in osteoclasts through activating the nicotinic receptors. The two blue-dashed arrows refer to possible effects of the PNS on osteoblasts and osteocytes.

itary-thyroid-bone axis^{3,8}. It has been reported that the expression of muscarinic receptors centrally might be regulated by the level of calcitonin⁶⁶, a hormone that plays a significant role in the pituitary-thyroid- bone axis. However, it has not been investigated whether this association between muscarinic receptors and calcitonin would affect bone mass.

Locus coeruleus

Muscarinic receptors, more specifically muscarinic-3 receptors, are expressed in non-adrenergic neurons in the locus coeruleus nucleus, a brain structure necessary for the SNS¹⁰. It has been shown that mice with knockout neural muscarinic-3 receptors are osteoporotic due to an increase in SNS signaling, indicating that muscarinic-3 receptors in the locus coeruleus nucleus down-regulate SNS signaling and favor bone mass indirectly¹⁰.

Central IL-1

Central IL-1 is a proinflammatory cytokine produced by brain neurons that are known to regulate learning, memory and sleep patterns^{67,68}. Central IL-1 can also affect bone metabolism through nicotinic nerve fibers of the PSNS¹¹. Indeed, mice with knockout central IL-1 express very low levels of skeletal acetylcholine¹¹, and these mice are osteoporotic due to the decrease of apoptosis in osteoclasts^{11,69}.

Pathways by which the cholinergic system regulates bone remodeling

Despite the substantial evidences we presented regarding the association between cholinergic activities at bone tissues-, neurons- and nuclei-level with bone remodeling, the pathways by which the cholinergic system affects bone remodeling are not well understood. It is still not known whether the cholinergic activity affects bone cells locally, systemically or both. In Figure 1, we suggest possible mechanisms for cholinergic regulation of bone. Recent studies have shown that cholinergic activity might regulate

bone mass systemically through neuronal pathways mediated parasympathetic activity^{10,11}.

Clinical observations

Cholinergic-regulated tissues

Cholinergic activity regulates many organs in which cholinergic receptors have been identified such as: salivary cells, lacrimal cells, langerhans cells, the respiratory system, the gastrointestinal system and the vestibular organ within the ear (Table 1)^{24,70-77}. Decrease cholinergic activity in salivary glands function results in a dry mouth (xerostomia)⁷⁸. Similarly, suppression of cholinergic activity in lacrimal glands results in dry eyes⁷⁹. Damage to muscarinic-3 receptors in langerhans cells result in diabetes type 1⁸⁰. Surprisingly, several articles in the literature have reported strong association of an unknown etiology between these medical conditions (xerostomia, dry eyes and diabetes type 1) and bone loss⁸¹⁻⁸⁴. However, the evidence we present in this study seems to indicate that bone loss in these medical conditions could be due to alterations in cholinergic activity.

Suppression of cholinergic activity is also known to cause the onset of serious pathologies such as obstructive pulmonary diseases, disruption in the body's circadian rhythm and learning memory deficits⁸⁵⁻⁸⁸. Interestingly, all these conditions have as a common skeletal feature, a reduction in bone mineral density^{85,89,90}. Another interesting condition which is associated with cholinergic activity is vertigo. Vertigo patients are characterized by dizziness and being off-balanced as a consequence of a damage to the vestibular end organ in the ear⁹¹, a cholinergic-regulated organ⁷⁶. Patients suffering from vertigo are associated with higher risk of osteoporosis⁹².

Circadian rhythm

An interesting phenomenon that links cholinergic activity with bone remodeling is the circadian rhythm. The autonomic nervous system follows a circadian pattern that matches the bone remodeling cycle. Sympathetic activity is dominant during day hours when bone resorption activity reaches its peak, while the parasympathetic activity is intense during night hours when bone formation is more active^{93,94}.

Menopause

Another observation that links cholinergic activity to bone is the estrogen replacement therapy, a hormonal treatment for osteoporosis. Patients under estrogen replacement therapy are well known to have high bone mass and low fractures risk⁹⁵. Surprisingly, these patients express an increase in cholinergic activity⁹⁶.

Smokers

Clinical studies have shown that heavy smoking is associated with a decreased bone mass and diminished fracture healing capacity^{31,97,98,99}. Even though many hypotheses have been postulated to explain this phenomena, it has been found that the association between smoking and bone is strongly related

to nicotine levels in the body¹⁰⁰. Even though low concentration of nicotine up-regulates osteoblasts through activation of the nicotinic receptors^{32,39}, excessive levels down-regulate osteoblasts through desensitizing their nicotinic receptors³⁸. Also excessive nicotinic levels up-regulate osteoclasts through activation of the nicotinic receptors¹¹.

Poliomyelitis, Botox injection and Myasthenia gravis

Cholinergic neurons innervate muscle fibers¹⁰¹, and their activity can affect bone mass through mechanical stress¹⁰². Indeed, it has been argued whether the muscle activity, rather than the neuronal activity, is the main regulator of bone mass. However, the following observations argue strongly against a model whereby the muscle activity is more important than neuronal activity in regulating bone mass. Underneath, we discuss how three conditions, known to affect neuro-muscular synapses: Poliomyelitis, Botox injections and Myasthenia gravis, affect bone health status

Poliomyelitis is a viral disease that destroys motor neurons that utilize acetylcholine neurotransmitter resulting in muscle paralysis¹⁰³. Patients diagnosed with poliomyelitis are known to suffer from impaired bone growth affected by nerve depletion¹⁰⁴. Interestingly, it has been found that after recovery of muscle activity, polio patients tend to develop osteoporosis in a much larger proportion than the rest of the population, indicating that the effect of polio virus on bone tissue is unrelated to muscle function¹⁰⁴.

Botox, known as botulinum neurotoxin, prevents the release of acetylcholine from its membrane vesicles at the terminal ends of cholinergic neurons resulting in muscle paralysis¹⁰⁵. Botulinum neurotoxin causes bone loss that does not improve following the recovery of muscle function^{106,107}. Moreover, bone loss due to botulinum neurotoxin has been associated with an increase in bone resorption due to osteoclasts up-regulation¹⁰⁶, a phenomena that is recently linked to the inhibited activity of cholinergic fibers of the PSNS¹¹. Accordingly, these findings indicate that the effect of botulinum neurotoxin on bone tissue is unrelated to muscle function.

Myasthenia gravis is a disorder that is characterized with suppressed muscular activity due to autoantibodies blocking the cholinergic receptors in muscle fibers. Surprisingly, despite the low muscular activity, patients diagnosed with Myasthenia gravis are not associated with the risk of developing bone diseases¹⁰⁸⁻¹¹⁰. In contrast, Myasthenia gravis patients are more resistant to develop osteoporosis compared to general population¹⁰⁹. Myasthenia gravis only affects acetylcholine receptors in muscles leaving cholinergic signaling in bone intact, which might explain why bone is not affected in these patients.

The linking findings described for the above three conditions indicated that cholinergic innervation is necessary and might be more important than muscle activity for healthy bone remodeling. However, future studies will have to be performed in order to confirm the role of cholinergic activity in defining the bone phenotype of patients with Poliomyelitis, Myasthenia gravis or following Botox injections.

Future therapies for osteoporosis

The available literature indicates that the inhibition of cholinergic activity at the bone level and in the central nervous system reduces bone mass^{10,11,40}. This has lead researchers to investigate whether boosting acetylcholine activity might have an anabolic effect on bone formation.

One way of stimulating cholinergic receptors is by the administration of cholinergic agonists such as acetylcholinesterase inhibitors (AChEIs). AChEIs are a group of drugs that cause stimulation of cholinergic receptors (nicotinic and/or muscarinic) by inhibiting the action of AChE and increasing the levels of acetylcholine in the synaptic space. A recent study has revealed that the use of pyridostigmine, a peripherally acting AChEI that stimulates nicotinic receptors, favors bone mass in animal models by stimulating osteoclast apoptosis¹¹. Another recent clinical study reported that treatment with centrally acting AChEI that stimulates both nicotinic and muscarinic receptors such as donepezil and rivastigmine was associated with lower risk of hip fracture in Alzheimer's disease patients¹¹¹. In the same study, it was shown that centrally acting AChEI that stimulates nicotinic receptors only such as galantamine had no beneficial effect in lowering the risk of hip fracture¹¹¹. Accordingly, it seems that stimulating cholinergic receptors through pharmacological approaches could favor bone mass. These findings open the window for a new therapeutic approach to treat osteoporosis through stimulation of the cholinergic system.

Future studies

Even though there is substantial evidence in the literature indicating the importance of cholinergic activity on bone, there is however still much to learn about the complicated relationships between the cholinergic system and bone. For instance, future studies will have to be performed in order to investigate whether the cholinergic activity affects bone mass by other mechanism rather than the IL 1-PSNS-bone and Locus coeruleus-SNS-bone pathways. The interaction between the cholinergic system with endocrine system, and the effects of this interaction on bone mass need to be investigated.

Another area that needs investigation is the possible involvement of nicotinic receptors expressed by osteocytes. It is well known that osteocytes can suppress osteoblast proliferation through the secretion of sclerostin, an inhibitor of the Wnt signaling pathway in osteoblasts, and promote osteoclast proliferation directly through RANKL secretion^{112,113}. Accordingly, nicotinic receptors in osteocytes could be mediating the interactions between these cells with osteoblasts and osteoclasts, although future studies will have to be performed to address this hypothesis.

Conclusion

In this review we have summarized several observations demonstrating that the cholinergic signaling is a positive reg-

ulator of bone mass. *In vitro* studies have suggested that acetylcholine might regulate proliferation and differentiation of bone cells. *In vivo* studies have shown that altering cholinergic activity regulates bone mass accrual. Clinical studies have shown that diseases caused by disruption of cholinergic activity seem to be associated with bone disorders. Even though we here presented substantial evidence from the literature indicating the importance of cholinergic activity on bone, however there is still much to learn about the complicated relationships between the cholinergic system and bone.

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